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Abundant class of non-coding RNA regulates development in the social amoeba *Dictyostelium discoideum*

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Non-coding (nc)RNAs are important players in most biological processes. Although small RNAs such as microRNAs and small interfering RNAs have emerged as exceptionally important regulators of gene expression, great numbers of larger ncRNAs have also been identified. Many of these are abundant and differentially expressed but their functions have in most cases not been elucidated. The social amoeba *Dictyostelium discoideum* contain the ncRNAs commonly found in eukaryotes. In addition, we previously reported the identification of two novel classes of 42–65 nt long stem-loop forming RNAs, Class I and Class II RNAs, with unknown function. In this study we have further characterized these abundant ncRNAs, which are downregulated during development. We have confirmed expression of 29 Class I RNAs and experimentally verified the formation of the computationally predicted short conserved stem structure. Furthermore, we have for the first time created knockout strains for several small ncRNA genes in *D. discoideum* and found that deletion of one of the Class I RNAs, DdR-21, results in aberrant development. In addition we have shown that this Class I RNA forms a complex with one or several proteins but do not appear to be associated with ribosomes or polysomes. In a pull down assay, several proteins interacting with DdR-21 were identified, one of these has two RNA recognition motifs (RRMs). The purified RRM containing protein was demonstrated to bind directly and specifically to DdR-21.

Introduction

In the last few years the number of new non-coding (nc)RNAs has increased enormously mostly due to the advent of high-throughput sequencing technologies. This has lead to the discovery of numerous members of the smallest classes of ncRNAs such as micro (mi)RNAs, small interfering (si)RNAs and Piwi interacting (pi)RNAs which have important regulatory functions in different eukaryotic organisms.[1] These and other regulatory RNAs normally act through ribonucleoprotein (RNP) complexes such as the RNA induced silencing complex (RISC) to affect their targets in various ways. There are however several classes of eukaryotic ncRNAs for which the biological functions are completely unknown or very elusive. Y RNAs, for example, were discovered as components of the Ro ribonucleoprotein (RNP) 30 years ago.[2] These RNAs have been suggested to be involved in RNA quality control[3,4] and to play a role in DNA replication.[5] Stem-bulge RNAs (sbRNAs) in *Caenorhabditis elegans* is another example.[6] The function of sbRNAs is yet uncharacterized but it has recently been suggested that they are homologs to vertebrate Y RNAs.[7] The Y RNAs and sbRNAs are just two examples of ncRNAs for which the presence in eukaryotic cells has been known for years but where a detailed understanding of their function is still lacking, emphasizing the difficulty in assigning biological roles for ncRNAs.

Dictyostelium discoideum is a social amoeba with an unusual life cycle, it feeds and divides as single cells but under conditions when food supplies are running low, up to 100,000 cells aggregate and form a multicellular structure.[8] *D. discoideum* belongs to the amoebozoa, a group of organisms evolutionarily positioned after the division of plants and animals but before the split of animals and fungi.[9] The organism has for long served as a model organism for a wide range of cellular mechanisms including motility, differentiation and development, phagocytosis, and more recently host-pathogen interactions.[10] The evolutionary position, the intriguing life cycle, and the ease of growing and manipulating *D. discoideum* makes it an interesting and useful model also for studying different aspects of ncRNAs. In 2004 we published the first extensive search for 50–500 nt ncRNAs in *D. discoideum*.[11] This study was followed up by further computational and experimental analyses to better understand the ncRNA landscape of *D. discoideum*.[12-15] Many of the well-known classes of ncRNA were identified although when these were studied in great detail several unexpected findings were revealed such

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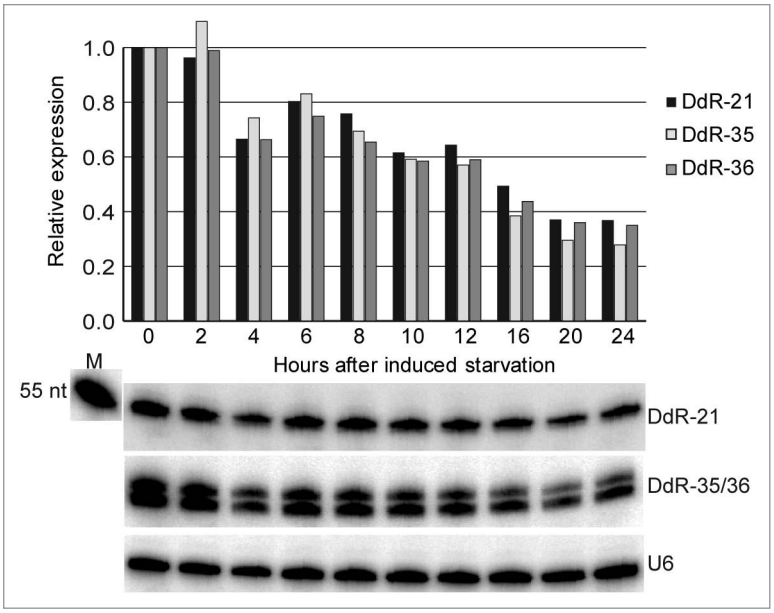

Figure 1. Class I RNA expression during development analyzed by northern blot. Time points represent hours after onset of starvation where 0 h depicts growing cells. Each part shows the same membrane successively probed for the different RNAs. The probe used for DdR-35 and 36 recognizes both RNAs. M denotes radioactively labeled 55 nt RNA. The expression is normalized to U6 snRNA (graphs at the top of the figure). RNA levels at 0 h are set to one.

as divergent spliceosomal RNAs (snRNAs) which were enriched in the cytoplasm and developmentally regulated.[12] In addition two entirely novel classes of ncRNAs, called Class I and Class II RNAs were identified. These RNAs are 42–65 nt long and the 5' and 3' ends are predicted to basepair to form a short stem structure (**Fig. S1**). In addition, an 11 nt conserved sequence element is located just downstream of the 5' part of the stem. These RNAs are present at high levels, mainly in the cytoplasm, and are downregulated during development. There are 42 predicted Class I and Class II genes in *D. discoideum* and at least 16 of these were shown to be expressed.[11] The genes are found on all six chromosomes and often appear in clusters. In particular, about half of the Class I RNA genes are situated on chromosome four where the majority are organized in three clusters[14] (dictybase.org/). All the expressed genes are preceded by the Dictyostelium upstream sequence element DUSE, predicted to constitute a promoter element for many ncRNA genes in *D. discoideum*.[11,12,14,15] Until recently no homologs to Class I or Class II RNAs had been found, partly because the sequence of other Dictyostelium genomes was not available. However, a very recent comparison of the draft genome of *Dictyostelium purpureum* and the *D. discoideum* genome revealed at least 26 homologs present in *D. purpureum*.[16] Although *D. purpureum* belongs to the same group of Dictyostelia as *D. discoideum*, they probably shared the last common ancestor about 400 million years ago.[17]

Class I and II RNAs share the 11 nt conserved stretch following the 5' part of the stem but the sequence of the stem differs and therefore we initially classified them as two separate classes. However, new data emerging from other Dictyostelia species illustrate that the stem sequences of Class I RNAs varies between different species while the 11 nt is well conserved[16] and (Avesson, Reimegård and Söderbom, manuscript in preparation). It is therefore more appropriate to divide the RNAs into subclasses and we here redefined *D. discoideum* Class I and Class II RNAs as Class Ia and Class Ib RNAs, respectively.

In this investigation, we have studied in more detail the function of Class I RNAs. We first created knockout strains for two different Class I RNA genes in *D. discoideum*. This is the first time that genes for short ncRNAs have been disrupted in this organism. One of the knockout strains exhibited aberrant development resulting in smaller fruiting bodies but at a higher number, giving us a first indication of the function of Class I RNAs. We have also investigated how the RNA interacts with other macromolecules in the cell and identified Class I RNA interacting protein(s). One of these, harboring two distinct RNA recognition motifs (RRMs), was shown to bind specifically to the RNA in vitro. In addition, our data suggest that the structure of Class I RNAs is important and that the individual RNAs may have, at least to some degree, overlapping functions. Hence, we have started to reveal the function for a specific class of ncRNAs in *D. discoideum,* which is also present in other Dictyostelia species. Furthermore, by demonstrating that ncRNA genes can be easily knocked out in this amoeba we strongly believe that *D. discoideum* will be of general use to understand the function of ncRNAs during growth and development.

Results

Expression of Class I genes. We have previously reported the construction of cDNA libraries representing full-length 50–500 nt RNAs of *D. discoideum* where two abundantly expressed new RNA classes, Class I and Class II RNAs were identified.[11] Here, we have re-defined Class II RNAs as a subclass of Class I RNAs, due to structural, sequence and expression pattern similarities. In particular, both subclasses of RNAs carry a conserved 11 nt long sequence motif as well as a conserved predicted and verified (see below) short base-paired stem. Although the nucleotide composition of the stem is essentially conserved within each subclass it differs between them (**Fig. S1**). Hence, Class I RNA and Class II RNA will from now on be specified as Class Ia RNA and Class Ib RNA, respectively.

In order to further investigate the expression and abundance of this class of RNA, deep sequencing libraries from different developmental stages (Avesson, Reimegård and Söderbom, manuscript in preparation) of small RNAs (10–50 nt) were analyzed for fragments of Class I RNAs.

Besides the 16 different Class I RNAs previously found to be expressed and predicted to be encoded from 19 loci, sequences of ten additional Class I RNAs were identified in the deep sequencing libraries (**Fig. S2**). Since most Class I RNAs are longer than 50 nt, the deep sequencing reads in most cases do not represent

the full length RNA. Interestingly, the majority of these reads start at the reported first nucleotide of the RNAs, confirming the 5' end of the mature Class I RNAs. The great majority of the reads derives from Class I RNAs that were previously identified in the full-length cDNA library (see above), which was far from being saturated (**Fig. S2**). Since different methods were used to generate the full-length cDNA library and the SOLiD libraries, the result indicates that these particular members are the most abundant Class I RNAs in the cell.

In conclusion, it appears as if at least 29 out of the predicted 42 Class I genes are expressed in *D. discoideum*. Of the remaining 13 predicted genes, eight may be expressed under different conditions while five most likely represent pseudogenes due to the lack of the predicted promoter element DUSE (data not shown).

Developmental regulation of Class I RNA genes. Class I RNA genes are collectively downregulated at two developmental stages, the slug stage and at the final structure, the fruiting body as compared to growing cells.[11] To increase the resolution of this analysis, RNA was collected every 2–4 hours throughout development and analyzed by northern blot (**Fig. 1**). The result clearly shows that the tested Class Ia RNA DdR-21 and the two Class Ib RNAs DdR-35 and DdR-36 are downregulated throughout development ending up at about 30–40% in the fruiting body as compared to growing cells.

Construction of Class I deletion strains. In order to understand the function of Class I RNA we developed a strategy to delete ncRNA genes in *D. discoideum*, based on an established method to knock out protein coding genes.[18] One possible problem when knocking out short ncRNA genes is the extremely high AT-content (86%) surrounding these genes.[15] We expected this to reduce the specificity of homologous recombination required for specific gene disruption. In addition, the high AT-content makes it difficult to design primers for PCR amplification of the genomic fragments required for the recombination event. These problems were addressed by anchoring the homologous sequences in protein coding regions which have about 72% AT-content,[9] i.e., the knockout constructs were designed to overlap with at least one neighboring protein coding gene (**Fig. 2**).

Two Class I RNA genes were targeted for deletion, DdR-21 and DdR-33.[11] In both cases the entire gene was removed together with some of the flanking region and replaced with a Blasticidin S resistance cassette (**Fig. 2**). Transformants were screened by PCR and the absence of DdR-21 RNA was verified by northern blot (**Fig. S3**). The lack of DdR-33 expression was also confirmed in this way (data not shown). Furthermore, the neighboring genes were sequenced to confirm that no mutations had been inserted during the recombination event. Hence, we have for the first time deleted short ncRNA genes in *D. discoideum*.

Phenotypic analyses of strains depleted of the Class I RNAs DdR-21 and DdR-33. Phenotypic consequences of depletion of the two ncRNAs were analyzed during growth and development. Deletion of DdR-21 did not result in any significant change in generation time when grown in axenic medium. Wild type and DdR-21 deletion strain were also grown in association with *Klebsiella aerogenes* to investigate if feeding on bacteria was altered but no differences in plaque formation could be observed (data not shown). To investigate if the absence of DdR-21 has any effect on development, cells were starved by removal of nutrient and spread on cellulose filters. The isogenic wild type strain underwent normal development, synchronously forming distinct multicellular structures during a 24 hours time course. However, the DdR-21 deletion strain formed more and smaller fruiting bodies than the wild type strain (**Fig. 3**). The developmental assay was repeated several times and we consistently observed the aberrant phenotype although with variation in severity. To confirm the result, we recreated the gene disruption and observed the same effect on development. We further asked the question if the deletion affected certain protein coding genes expressed at specific stages of development, indicative of irregular cell differentiation. Northern blot analyses of aggregation, prestalk and prespore specific gene expression, i.e., *acaA*, *ecmA* and *cotB* showed that these genes were expressed at the same level and time as in wild type cells indicating that early cell differentiation was not influenced by the absence of DdR-21 (data not shown). Cells deleted for the Class I DdR-33 gene did not differ from wild type cells under the described experimental conditions (data not shown).

These results show that at least one of the Class I RNA members function in development by affecting the size and number of multicellular structures. Furthermore, from northern blot and SOLiD data (**Fig. S2** and data not shown) it appears as if DdR-21 is one of the most highly expressed Class I RNAs. The high

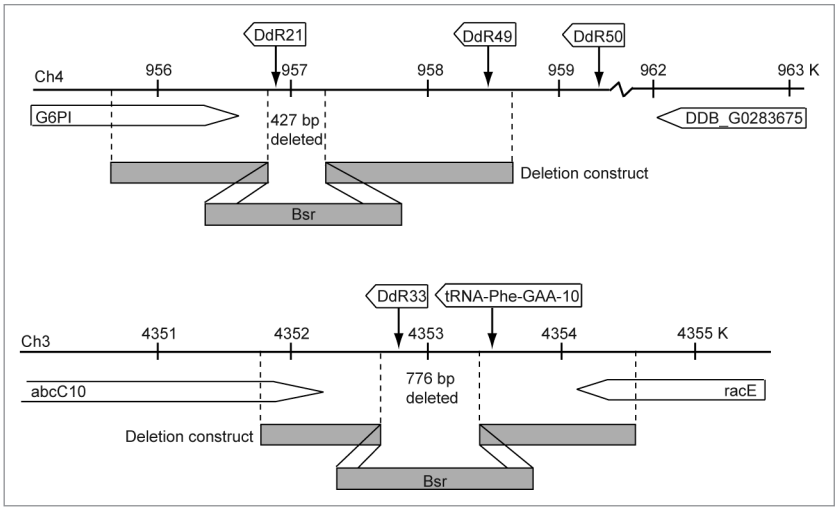

Figure 2. Schematic drawing of the construction of strains deleted for the genes encoding Class I RNAs DdR-21 and DdR-33. Integration sites and surrounding regions of Chromosome 4 and 3, Ch4 and Ch3, respectively, are shown with numbers representing kilo base pairs. The number of base pairs deleted as a consequence of the constructions is depicted. Open arrows below the chromosome line represent protein genes surrounding the deletions and open arrows above indicate ncRNA genes, not drawn to scale (http://dictybase.org/). Deletion constructs are shown in grey where the homology regions are surrounding the Blasticidin S resistance cassette (Bsr).

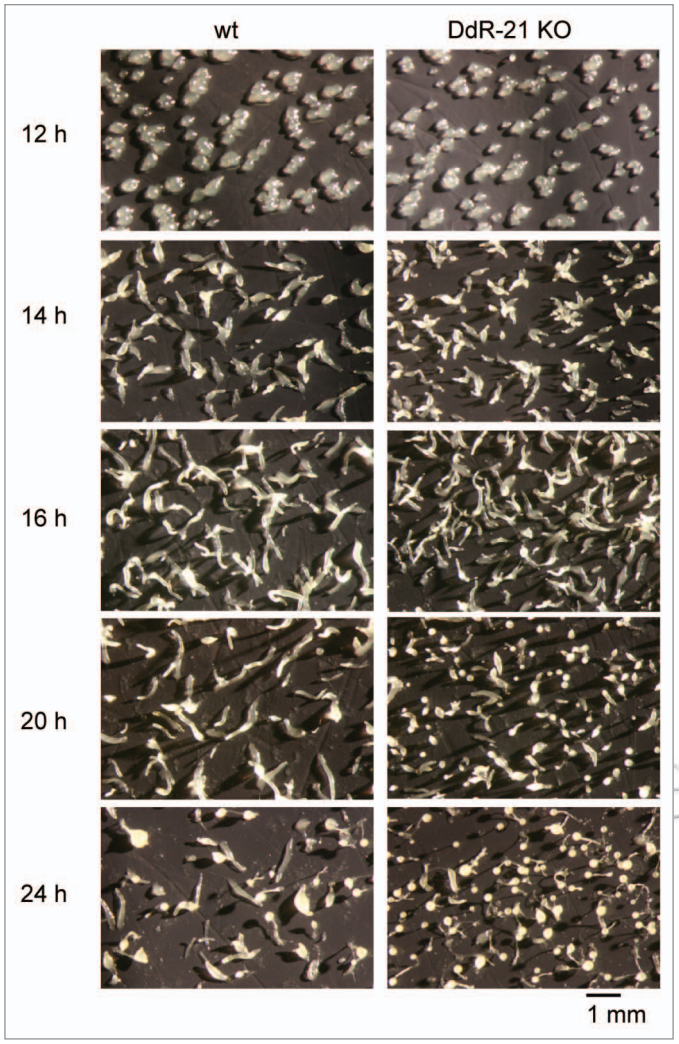

Figure 3. Phenotypic analysis of DdR21 knock out (KO) strain compared to wt during development. Time points represent hours after onset of starvation.

expression could be a reason why a phenotype is observed when deleting DdR-21 but not DdR-33, which is expressed at a lower level (**Fig. S2** and data not shown).

Analysis of Class I RNA structure in vitro. It is well accepted that the structure of ncRNAs often is important for their function.[19] All Class I RNAs have conserved sequences in the 5'- and 3'-ends, computationally predicted to basepair to form a six bp stem structure. The rest of the RNA is predicted to take a more open structure.[11] In order to analyze the secondary structure of Class I RNA DdR-21 in vitro, we performed in-line probing as well as chemical and enzymatic probing. In-line probing is dependent on the spontaneous cleavage of RNA by intramolecular phosphoester transfer, which occurs faster in single stranded regions due to higher degree of flexibility.[20] **Figure 4** shows in-line probing using DdR-21 RNA labeled at either the 5' or 3' end. The 5' and 3' ends are protected from spontaneous cleavage suggesting that they are present in a double stranded conformation. These data correlate with the accessibility of the double-stranded region towards RNase V1 (**Fig. S4B and C**) which cleaves paired or stacked regions of the RNA independent of sequence.[21] The in-line probing is also in agreement with our experiments where single stranded RNA was analyzed by chemical probing (**Fig. S4A and B**), i.e., reactivity of N1 position of adenine and N3 position of cytosine towards DMS and N3 position of uracile and N1 position of guanine towards CMCT.[21] Indeed, nucleotides which are highly reactive to modification by DMS or CMCT are mainly located in loop regions. The most accessible nucleotides in the loop harboring the conserved 11 nt motif are at position 8–11, 15–17 and 22–23. The presence of RNase V1 cuts validates the existence of a stem-loop structure encompassing nucleotides 24–44. However, the weak reactivities of nucleotides toward chemicals show that the helix is rather flexible. Modified bases are identified by primer extension, which is why we used DdR-21 RNA extended by a 30 nt 3' tail (DdR-21 + 3' tail) to which a primer was annealed. RNase V1 cleavage was used to exclude that presence of the 3'-tail changed the structure of DdR-21 (data not shown). **Figure S4** shows the most representative autoradiograms of chemical and enzymatic probing, based on several experiments. Taken together, the structural probing supports basepairing of the 5' and 3'-end as well as the relative open conformation of the loop with the conserved 11 nt sequence motif. The results also confirm the predicted stem-loop structure at the apex of the RNA which seems to be rather flexible. Similarly to the conserved stem connecting the 5' and 3' ends of the RNA, the existence of the upper helix is predicted to be conserved in all Class I genes, although with varying lengths (**Fig. S2**).

Class I RNA DdR-21 is not substantially associated with ribosomes or polysomes. ncRNAs generally form complexes with other RNAs and/or proteins in the cell. In order to investigate the possible complexes Class I RNAs participate in, cell extract was separated on sucrose gradients (**Fig. 5**). Class I RNAs were previously shown to be localized mainly to the cytoplasm.[11] We therefore used a gentle method to prepare cell extract by pressing cells through a filter. This will in most cases not disrupt the nucleus and will also keep other compartments intact in order to reduce RNA degradation. The cytoplasmic extract was fractionated on a sucrose gradient and the RNA was extracted from the different fractions and analyzed by northern blot (**Fig. 5**). The blot was probed for five different RNAs; DdR-21 (Class Ia RNA), DdR-35/36 (Class Ib RNAs), signal recognition particle (SRP) RNA, 5S rRNA and actin mRNA. 5S rRNA is expected to be mainly associated with the ribosomes (large peak at A260) and mRNA with polysomes (fractions with high sucrose concentration). The result showed that DdR-21 RNA is primarily present in fractions with low sucrose concentration corresponding to low molecular weight molecules and is most abundant in fractions where the SRP RNA is detected. SRP RNA is expected to be enriched in ribosomal fractions as seen for the tryptanosomatid *Leptomonas collosoma*.[22] We do not observe this in our experiment where the gradient was prepared with 150 mM KAc. It is possible that a lower salt concentration is necessary to maintain SRP binding to ribosomes. We therefore repeated the experiment in the presence of 25 mM KAc and could observe slightly increased levels of SRP RNA in ribosomal fractions. However, the profiles of DdR-21 and DdR-35/36 did not shift in spite of the lower salt

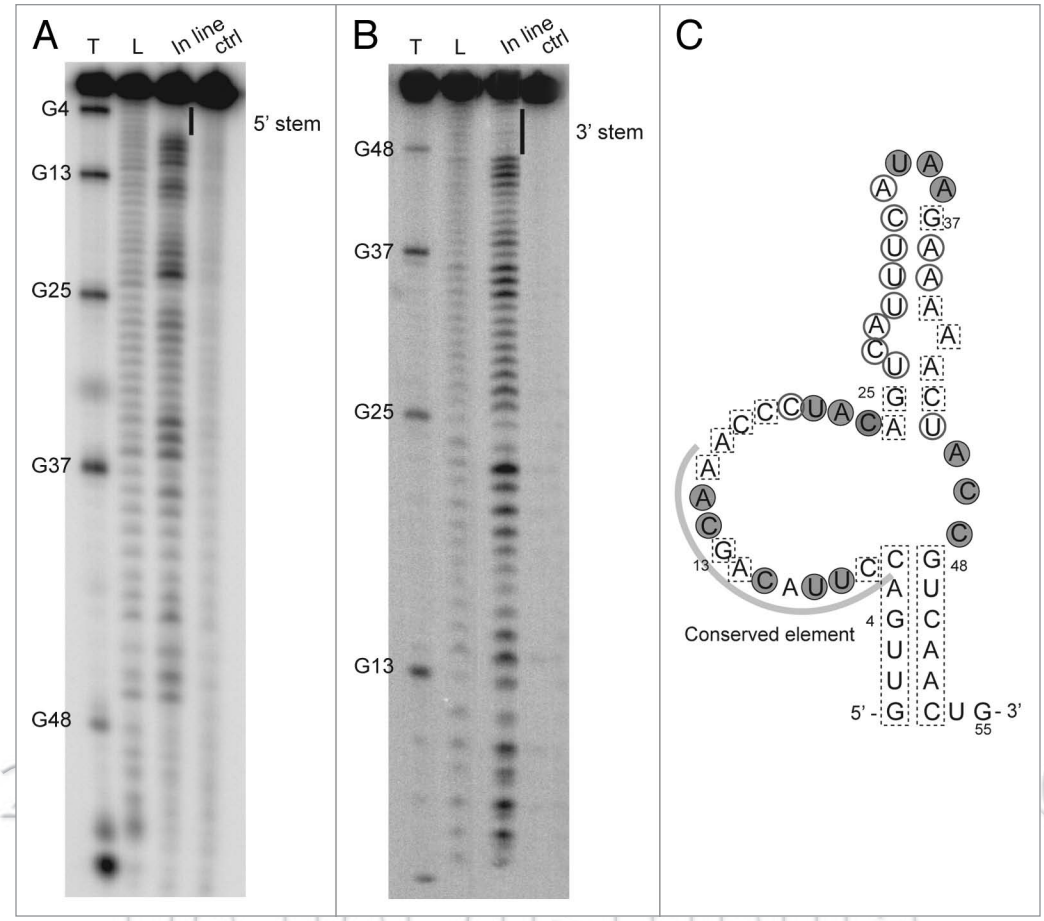

Figure 4. Determination of the in vitro structure of DdR-21. In-line probing of 3′ end labeled (A) and 5′ end labeled (B) in vitro transcribed DdR21 RNA. Lanes T and L represent RNA cleaved by RNase T1 under denaturing conditions and alkaline ladders of end labeled RNA, respectively. Ctrl depicts untreated labeled RNA. Solid lines marked 5′ stem and 3′ stem indicate the 5′ and 3′ nucleotide sequences, respectively, forming the base-paired stem drawn in (C). (C) Schematic drawing showing the consensus cleavage pattern. Filled circles represent nucleotides that are subject to strong cleavage, open circles represent moderately cleaved nucleotides and dashed squares weakly or not cleaved nucleotides. The conserved 11 nt sequence is indicated by a solid line.

concentration (data not shown). These results strongly suggest that Class I RNAs are not associated with ribosomes or polysomes to any large extent, but may form complexes with other partners in the cell. It should be noted that small amounts of both SRP RNA and Class I RNA are present at a very low level in lower fractions of the gradient. We suspect that this is due to minor contamination during fractionation and probably do not represent true associations with ribosomes or polysomes.

Class I RNA DdR-21 associates with cytoplasmic protein(s). The slight retardation of Class I RNAs in the sucrose gradient suggests that the RNA is present in a specific complex. To further study these Class I RNA interaction partners, we performed electrophoretic mobility shift assays (EMSA) where in vitro transcribed radioactively labeled DdR-21 RNA was mixed with cytoplasmic cell extract. A clear shift was consistently observed in the presence of cell extract (**Fig. 6**). The shift disappeared when proteinase K was added to the reaction indicating interaction with one or several proteins (data not shown). The interaction between DdR-21 RNA and protein(s) is fairly specific since a much higher concentration of tRNA as compared to unlabeled DdR-21 was required to out compete the interaction (**Fig. 6**). Since the crude extract naturally contains RNases it was not possible to avoid substantial degradation of the labeled RNA. Addition of RNase inhibitors did not have a marked effect, except in the case of ribonucleoside-vanadyl complex. However, although efficient in preventing RNA degradation, ribonucleoside-vandyl complex also inhibited the RNA-protein complex formation completely (data not shown).

In conclusion, the data suggest that Class I RNA DdR-21 binds to protein(s) in the cytoplasm and that the interaction is specific, or at least more specific than the interaction with tRNAs.

Identification of interacting proteins. To identify the protein(s) that interact with DdR-21 RNA a pull down assay was carried out. Biotinylated DdR-21 RNA was immobilized on streptavidin sepharose beads and used to pull out proteins from cytoplasmic cell extract. After washing, bound proteins were eluted and analyzed by SDS PAGE. Eluted proteins, that were not present in control reactions, were analyzed by mass spectrometry (**Fig. 7**). One of the most prominent band from several independent experiments turned out to be a protein with two

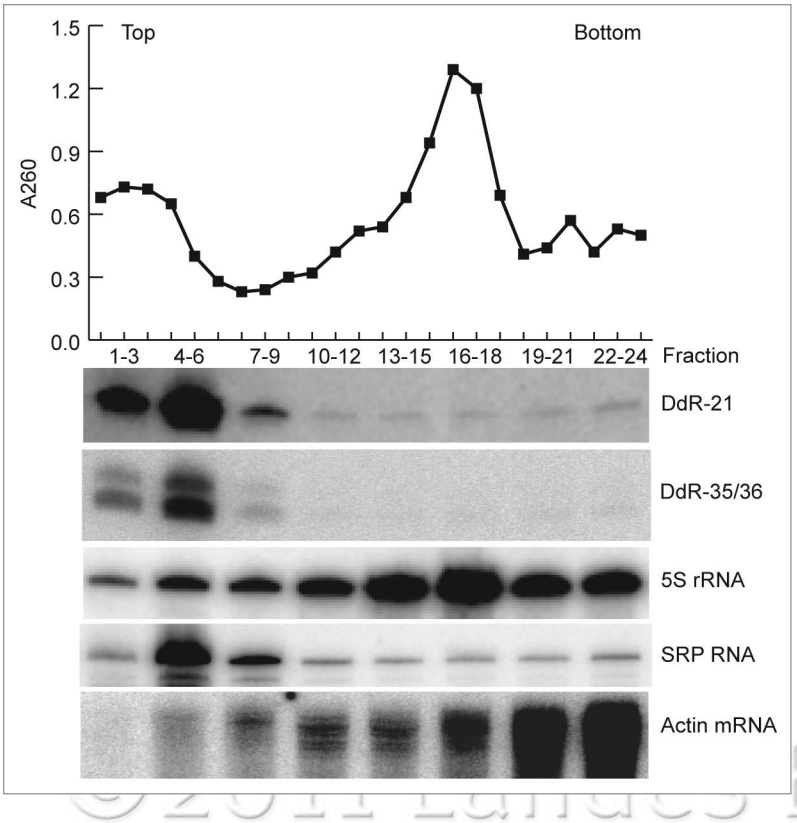

Figure 5. Cytoplasmic cell extract separated on 5–50% sucrose gradient. 5% indicate top and 50% bottom of the gradient, respectively. RNA content of each fraction was determined by measuring A260. Fractions were pooled three and three and analyzed by northern blot. Expression of different RNAs was analyzed using specific probes for the Class I RNA DdR-21 and DdR-35/36, 5S rRNA, SRP RNA and actin mRNA. Each part shows the same membrane successively probed for the different RNAs. The probe used for DdR-35 and 36 recognizes both RNAs.

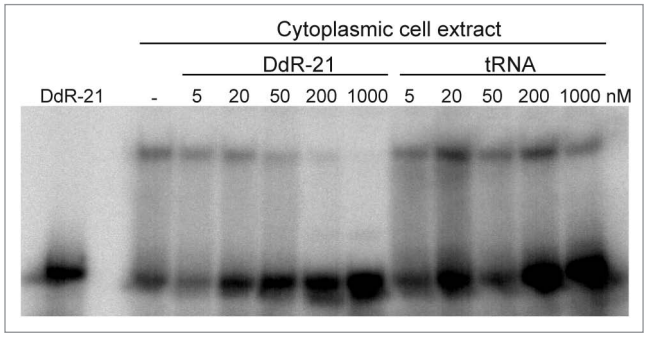

Figure 6. Electophoretic mobility shift assay with cytoplasmic extract on native 6% gel. 5′-end-labeled in vitro transcribed Class I RNA DdR21 was incubated alone or with cytoplasmic cell extract. The shift is competed with increasing concentration (5–1,000 nM) of unlabeled DdR21 and, to a much lesser extent, with tRNA, respectively. (-) depict the reaction without competitor.

RNA recognition motif 1 (RRM1) domains (labeled 2 in **Fig. 7**). The protein, DDB_G0284167, is annotated as a 31.8 kDa RNA recognition motif-containing protein (RRM) to which no function has been assigned (http://dictybase.org/). Although the PAGE indicate that the protein migrates as a protein of larger size, the heterologous expressed protein showed basically identical migration characteristic (see below and data not shown). The RNA binding protein is fairly short (293 aa) and consists of two 72 aa long RRMs separated by approximately 110 aa sequence without similarity to any known motifs. RRM motifs are very frequent and have been shown to bind both single and double stranded RNA as well as DNA and even proteins although binding to 2–8 nt of ssRNA is most common.[23] RRM1 containing proteins usually contain several RRM1 domains and it seems like multiple motifs increase the specificity of the RNA binding. The other proteins pulled down by DdR-21 include two enzymes involves in nucleotide synthesis such as inosine-5'-monophosphate (IMP) dehydrogenase (DDB0230098) and nucleoside diphosphate kinase (DDB0238334), and a Hypothetical protein (DDB0349338) (http://dictybase.org/).

Electrophoretic mobility shift assay of Class I RNA with purified protein. In order to investigate the binding between DdR-21 and the associated protein with two RRM1 motifs (from hereon called CIBP for Class I binding protein), the cDNA for CIBP was cloned and sequences encoding N- or C-terminal His-tags were added. The protein was expressed in *Escherichia coli* and purified by affinity chromatography. Gel shift experiments were performed with the purified CIBP using the same assay as with the cytoplasmic cell extract. The observed shift was out competed with much lower concentration of cold DdR-21 than with tRNA (**Fig. 8**) which is in line with the result from the EMSA where DdR-21 interacted with cytoplasmic protein(s) (**Fig. 6**). We were not able to analyze precisely the KD value due to nucleic acid contamination to the protein during expression in *E. coli* which could not be removed during purification (see Material and Methods).

Discussion

Non-coding RNAs have been studied for many years but their biological role have in many cases been difficult to reveal and the majority still awaits functional assignment. In this report, we have performed an in depth study of an abundant class of ncRNAs in *D. discoideum*, the Class I RNAs.[11,24] Class I RNAs are expressed at high levels in both growing and developing cells. Estimations from our northern blot data suggest that their expression is similar to that for the spliceosomal RNAs. According to high-throughput sequencing data, at least 29 out of 42 predicted Class I RNA genes are expressed and the level of expression seems to differ between the members (**Fig. S2**). The importance of Class I RNAs is emphasized by the fact that they are present in many Dictyostelia species. 26 Class I genes were recently computationally predicted in *D. purpureum*[16] belonging to the same Dictyostelia group (group 4) as *D. discoideum*.[25] In addition, putative Class I genes were predicted in *Dictyostelium*

citrinum which is closely related to *D. discoideum*, and also in *Polysphondylium violaceum*, a more distant species.[16]

Computational prediction, structural probing, and presence of compensatory basepair changes strongly suggest that formation of the stem connecting the 5'- and 3'-ends is essential but that the sequence of the stem can vary. This is demonstrated by Class Ia and Class Ib where the sequence of the stems differs between the two subclasses. This is also supported by our study including several Dictyostelia species (Avesson, Reimegård and Söderbom, manuscript in preparation) and the prediction of Class I RNAs in *D. purpureum*.[16] The 11 nt following the 5' part of the stem is almost perfectly conserved in the Dictyostelia species analyzed (see above) and seems to be in a relatively open conformation available for interactions with other molecules (**Figs. 4 and S4**). This region probably serves as some kind of recognition motif, either as a binding site for interacting proteins or for recognition of targets in form of other nucleic acids (see below).

In order to get a handle on the function of Class I RNA, we constructed deletion strains for two different Class I genes. This is the first time short ncRNA genes have been deleted in *D. discoideum*. The strain where the gene encoding Class I RNA DdR-21 had been knocked out ended up in smaller fruiting bodies which appeared at a higher number as compared to the wild type strain. Although the aberrant phenotype is rather mild, it is distinct and consistent. The other deletion strain where the Class I gene DdR-33 had been knocked out did not show any different behavior as compared to wt cells. The fact that only a mild phenotype was observed for one of the Class I deletion strains is not very surprising. Since many Class I RNAs are very similar in sequences it is possible that they have overlapping functions and maybe even regulating the same processe(s) where they only have a fine tuning effect. One class of ncRNA with such features is small nucleolar RNA (snoRNA), present in archea and eukaryotes where the member RNAs guide chemical modifications of other RNAs such as rRNA (reviewed in ref. 26). When a cluster of seven snoRNAs was deleted in yeast no obvious phenotype was observed.[27] Similarly, we have deleted two clustered snoRNA genes in *D. discoideum* without any apparent effect on growth or development (data not shown). Furthermore, several stem bulge RNAs (sbRNAs) in *C. elegans* have been knocked down without any visible phenotype.[28,29]

Another approach to reveal the function of ncRNAs is to find their binding partners. Hence, identification of other molecules that interact with the Class I RNAs could give clues toward their biological role. Electrophoretic mobility shift assay revealed that DdR-21 RNA can form a complex with one or more proteins. This was confirmed by pull down experiments, using Class I RNA DdR-21 as bait, where several proteins were isolated. One of these has predicted RNA Recognition Motifs (RRMs) at its N- and C-terminal ends and hence is a good candidate for a direct interaction partner with the Class I RNAs. The fact that RRM containing proteins can bind both single and double stranded RNA as well as DNA makes it very difficult to decipher their function and mechanistic role.[23] One or multiple RRMs are present in a large number of eukaryotic proteins. Searching the Pfam database[30] for other proteins containing two RRMs

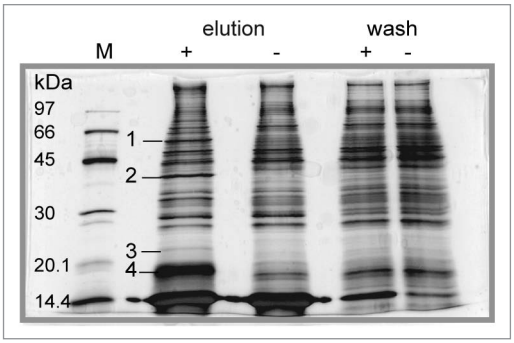

Figure 7. Pull down assay to isolate DdR-21 interacting proteins. Proteins from cytoplasmic extracts mixed with biotinylated Class I RNA DdR-21 immobilized on streptavidin sepharose beads (+). (-) depict control reactions where the extract was incubated with beads without DdR-21 attached. Wash depicts samples from the first washing step. Interacting proteins were eluted and analyzed by SDS PAGE. Protein bands labeled with 1–4 were analyzed by mass spectrometry. 1: IMP dehydrogenase (DDB0230098), *guaB* (DDB_G0283701); 2: RNA recognition motif-containing protein RRM (DDB0233340), DDB_G0284167; 3: Hypothetical protein (DDB0349338), DDB_G0281243; 4: Nucleoside diphosphate kinase (DDB0238334), *ndkC-2* (DDB_G0273069) (http://dictybase.org/).

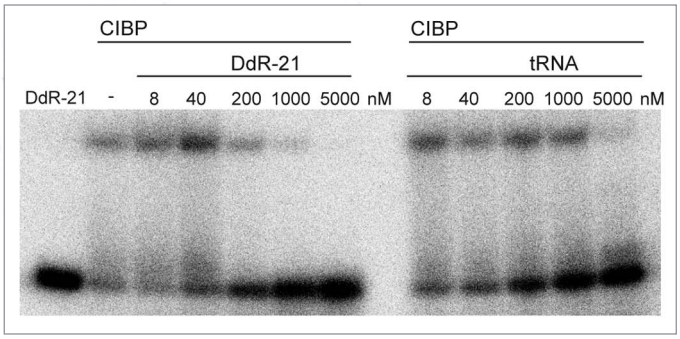

Figure 8. Electophoretic mobility shift assay with purified CIBP on native 6% polyacrylamide gel. 5′-end-labeled in vitro transcribed Class I RNA DdR21 was incubated with purified CIBP. The shift was competed with increasing concentration (8–5,000 nM) of unlabeled DdR21 and tRNA, respectively. (-) depict the reaction without competitor.

identify for example splicing factor U2AF and polyA-binding proteins. The sequence between the two RRMs in the isolated *D. discoideum* protein share no similarity to other domains and does not give any further clues to the function of the protein. We made several attempts to knockout the gene encoding the RRM containing protein but failed, which could indicate that the protein is essential. A synthenic ortholog is found in *D. purpureum* (DPU_G0071262) (http://dictybase.org/). It is annotated as a protein with only one RRM but our analysis of the downstream sequence suggests that the protein actually is longer and contains the second RRM as well (data not shown). In addition to the RRM containing protein, three other proteins were pulled down. Interestingly, two are involved in nucleoside synthesis.

Where in the cell would we expect Class I RNAs to exert their function? We have previously shown that Class I RNAs are mainly cytoplasmic,[11] hence it is unlikely that the RNAs

are directly involved in replication, transcriptional or epigenetic regulation. Furthermore, at least the Class I RNAs tested are not associated with ribosomes or polysomes to a high extent, indicating that they are also not directly involved in regulation of translation. It is however possible that Class I RNAs have a transient interaction with the ribosome. This would be analogous to SRP RNA, which we only detect at low levels in the ribosomal fraction of our sucrose gradient despite its function in translation translocation.[31]

With what mechanism does Class I RNAs regulate development? Many ncRNAs, for example snoRNAs and miRNAs, function as guides that direct effector proteins to their target nucleic acids via complementary basepairing. However, a similar mechanism for Class I RNAs is hard to envision. The variable sequence of Class I RNAs seems too heterogeneous and AT-rich to have distinct targets and we have failed to identify targets with continuous complementary sequences. Although we can not completely rule out that only short complementary regions are required for binding in a miRNA seed-like fashion. Another possibility is that Class I RNAs, instead of interacting directly to RNA targets, bind specifically to certain proteins and regulate their activity. There are several examples of ncRNAs acting in this fashion, i.e., as "molecular sponges". The long ncRNA MALAT1 have been shown to interact with splicing factors and influence the distribution and level of active factors.[32] Another example is B2 RNA in mouse, which can control transcription by preventing the contact between RNA polymerase II (Pol II) and promoter DNA and also by repressing the phosphorylation of Pol II.[33] It is possible that Class I RNAs have a similar function where the RNA titrates the CIBP protein and thereby inhibits its function, i.e., binding to other nucleic acids.

One hypothetical target for Class I RNA regulation would be the process involved in the control of cell counting. The size of aggregates, subsequently leading to fruiting bodies, is regulated by a secreted multisubunit counting factor (CF). CF in turn is controlled by a novel protein encoded from the *smlA* gene. Disruption or antisense knock down of *smlA* leads to large number of small fruiting bodies due to over-secretion of CF (reviewed in ref. 34). This phenotype is very similar to the one we observe when cells have been depleted of DdR-21. One model is that in cells depleted of Class I RNA DdR-21, some CIBP is released and can bind to the SmlA mRNA and downregulate the protein output either by inhibiting translation or induce degradation. This would lead to more secreted CF and hence a larger number of smaller fruiting bodies. Although there are no direct experimental data that support this hypothesis, it may serve as a working model for further analyses. It is also possible that Class I RNA function as a larger RNP complex, including the other isolated proteins, effecting nucleotide metabolism. However, the observed effect on development when cells are depleted of DdR-21 is more difficult to explain in relation to aberrant nucleotide synthesis.

In conclusion, we have used biochemical and genetic tools to analyze the function of a class of abundantly expressed RNAs, Class I RNA. This class of 42–65 nt long RNAs is downregulated during development, mainly located in the cytoplasm but not substantially associated with polysomes or ribosomes. However, Class I RNAs interact with other protein(s) in the cell, one of which has two RNA recognition motifs but with no other apparent functional domains (termed CIBP). We confirmed, in vitro, the computationally predicted Class I structure where the 5' and 3' ends form a short stem structure followed by, at the 5'-part, an 11 nt conserved sequence that appears to be in a more open conformation. The conserved sequence and/or structure is/are likely binders for the CIBP protein. Furthermore, cells depleted of one very highly expressed member of Class I RNA showed aberrant development manifested as smaller and more fruiting bodies. Hence, and importantly, we have shown that Class I RNA, at least DdR-21, has a function during development. This is the first time knocking out short ncRNA genes have been performed in *D. discoideum*. We anticipate that this will be a valuable tool also for researchers studying ncRNA in other organisms where gene knockouts cannot be performed. If homologous ncRNA/classes of ncRNAs of interest are present in *D. discoideum*, the genes can be disrupted and the phenotype, e.g., motility, growth, phagocytosis, cell differentiation and development, can be studied.

Material and Methods

Strains and growth conditions. Axenically growing *Dictyostelium discoideum* cells, AX4 and derivatives,[35] were cultivated in HL5 medium and developed in PDF buffer on nitrocellulose membranes at 22°C.[36] In some experiments, *D. discoideum* cells, were grown on SM plates in association with *Klebsiella aerogenes*.

Oligonucleotides. Sequences of DNA-(Invitrogen) and RNA-(Dharmacon) oligonucleotides used in this study are presented in **Table S1**.

Mapping SOLiD reads to Class I RNAs. Three libraries of short RNAs from axenically growing cells and developmental stages 16 hours and 24 hours with a length between 10–70 nt were sequenced using the SOLiD system generating single end reads with a length of 50 nt. Briefly, total RNA was isolated using Trizol (Invitrogen), size fractioned by FlashPage (Ambion) and prepared for sequencing using the SOLiD small RNA expression kit (Applied Biosystems) and finally sequenced using SOLiD3 (Applied Biosystems). The RNA fractionation and sequencing were performed at Uppsala Genome Center, Rudbeck Laboratory. Reads were mapped to a reference set of Class I (Class Ia) and Class II (Class Ib) RNAs in *D. discoideum* using SOLiD 1.3. The reference set includes both experimentally verified and bioinformatically inferred Class Ia and Class Ib RNAs.[11] Reads were allowed to map to the reference sequence in both directions to assess the false discovery rate. If a read was mapped to multiple Class I RNAs only the hits with the best score was kept for further analysis. In cases where a read mapped to multiple Class I RNAs with an identical score all hits were considered for further analysis.

Deletion of the genes for DdR-21 and DdR33 Class I RNA. Deletion constructs were assembled using pLPBLP,[18] containing the Blasticidin S resistance cassette, as host vector. Regions about 1,000 bp flanking the Class I genes were amplified by PCR from genomic DNA using primers 127 + 128 for the 5' region and 129

+ 130 for the 3' region for DdR-21 and primers 131 + 132 (5' region) and 133 + 134 (3' region) for DdR-33. The 5' region was first cloned into the Hind III site of pLPBLP. Subsequently the 3' region was cloned using the Pst I and Spe I sites. The 5' region of the DdR-21 deletion construct is anchored in the Glucose-6-phosphate isomerase gene and DdR-33 is anchored in the *abcC10* and *racE* genes. Deletion vectors were excised from the vector using restriction enzymes NotI, PvuI and EcoRI and gel purified. 10–30 μg linerarized DNA was transformed essentially according to reference 37. 10^7 *D. discoideum* AX4 cells were collected by centrifugation at 300x g and resuspended in 1 ml cold Zap buffer (10 mM NaH_2PO_4 pH 6.1, 50 mM sucrose) before electroporation. Selective media containing 5 mg/ml Blasticidin S was added to the transformants after 24 h. Colonies were collected 8–10 days post transformation and single clones were isolated on *K. aerogenes* lawns and analyzed by PCR using three different primer pairs, 154 + 128, 15 + 127 and 15 + 154 for DdR-21 and 132 + 155, 15 + 131 and 15 + 155 for DdR-33. Northern blot was used to confirm that the RNAs were not expressed.

In vitro transcription of RNA. DdR-21 and DdR-21 + 3' tail RNA was generated by in vitro transcription using MEGAscript (Ambion) with template obtained by annealing overlapping oligonucleotides 180 and 209 (DdR-21) or 284 and 285 (DdR-21 + 3' tail, template with 30 nt 3' tail) followed by PCR amplification using oligos 254 and 255 or 256. RNA was purified by PAGE on 8% polyacrylamide/7 M urea/1x TEB gels, eluted in (0.5 M NH_4OAc, 1 mM EDTA, 0.1% SDS) overnight at 4°C during shaking. The RNA was phenol/chloroform extracted, precipitated with EtOH and finally resuspended in sterile H_2O. Before each experiment all RNAs were renatured by incubation at 95°C for 1 min in H_2O followed by 1 min on ice and then 15 min incubation at 22°C (which is the temperature used for growth and development of *D. discoideum*) in the appropriate buffer (see below).

Chemical and enzymatic probing of DdR-21. Determination of the secondary structure of DdR-21 was performed by chemical modifications, enzymatic hydrolysis and In line probing. Chemical modifications of C(N3) and A(N1) with Dimethyl sulfate (DMS) were performed on 2 pmol of DdR-21 + 3' tail RNA in 20 μl native buffer (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 150 mM KCl) in presence of 2 μg tRNA. The reaction was started by addition of 2 μl DMS (diluted 1/8 or 1/2 in EtOH) followed by incubation for 10 minutes at 20°C. Modifications of U(N3) and G(N1) with 1-cyclohexyl-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate (CMCT) were performed in the same way but with a slightly different buffer (50 mM Borate-NaOH pH 8, 5 mM MgAc, 150 mM KOAc) followed by addition of 2 or 5 μl CMCT (60 mg/ml) and incubation for 20 min at 20°C. Enzymatic hydrolysis of DdR-21 + 3' tail RNA using 0.05 U RNase V1 was performed according to reference 38.

All reactions were stopped by addition of 0.3 M NaAc and 3 volumes EtOH. Modified sites or cleaved sites were detected by primer extension. Modified RNA was mixed with 0.4 pmol 5'-labeled oligo (255), samples were denaturated at 90°C for 1 min, cooled on ice and then incubated at 20°C for 5 min. Incubation was continued at 20°C for 10 min in RT buffer (50 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 50 mM KCl). Reverse transcription was subsequently performed with 0.3 mM dNTPs and 0.1 U/μl RT (AMV) in RT buffer at 37°C for 30 min. The RNA template was then destroyed by addition of 0.5 M KOH and 1 volume 50 mM Tris-HCl pH 8, 0.5% SDS, 7.5 mM EDTA and incubation at 90°C for 3 min then 37°C 1 h. RNA was precipitated with 0.4 M acetic acid, 0.3 M NaAc and 3 volumes EtOH. Samples were run on a 12% polyacrylamide/7 M urea/1x TEB gels and analyzed by autoradiography.

In line probing was performed on end labeled DdR-21 RNA. RNA for 5' end labeling was dephosphorylated using calf intestinal phosphatase (CIP/Fermentas) and labeled with (γ-^{32}P) ATP (PerkinElmer) using T4 PNK (Fermentas). RNA was 3' end labeled with [^{32}P] pCp (PerkinElmer) using T4 RNA ligase (Fermentas). The labeled RNA was purified by PAGE as stated above.

In line probing assays on 5' and 3' labeled DdR-21 RNA were performed according to reference 39. Briefly, RNA was incubated in 1x In line buffer (50 mM Tris HCl pH 8.3, 20 mM $MgCl_2$, 100 mM KCl) for 42 hours at 22°C and then analyzed on a 10% polyacrylamide/7 M urea/1x TEB gel.

Pull down of proteins binding to DdR-21. Cell extract from growing AX4 cells was prepared by collecting 5 x 10^7 cells by centrifugation at 300x g for 5 min, washing the cells once in PDF whereafter the cell pellet was resuspended in 500 μl filter lysis buffer (Hepes KOH pH 7.9, 5 mM MgAc, 250 mM sucrose). Cells were then passed through a 3 μm nucleopore polycarbonate membrane (Whatman). The lysate was centrifuged for 3 min at 13,000x g to remove cell debris. Total protein concentration was determined by Bradford assay (Biorad). Four-hundred microliters of Streptavidin Sepharose High performance Beads (GE Healthcare) was prepared by washing with 10 volumes of Buffer A (5 mM MgOAc, 20 mM Hepes KOH (pH 8), 0.1% NP40, 0.5 mM DTT, 150 mM KAc) followed by resuspension in Buffer A (1:1). In order to reduce the number of proteins binding unspecifically to the beads during the pull-down, crude cell extract (3 mg) was pre-incubated with 400 μl beads in buffer A for 5 min at 22°C and then 45 min at 4°C with rotation followed by centrifugation at 13,000x g for 20 sec. The supernatant was used for the pull-down experiments (see below). The bait RNA, 1,000 pmol biotinylated DdR-21 RNA (Dharmacon), was immobilized on 200 μl beads in buffer A for 45 min at 4°C with rotation followed by centrifugation at 13,000x g for 20 sec whereafter the supernatant was discarded. The pre-treated extract and the immobilized RNA were mixed and incubated for 15 min at 4°C after which 20 μg yeast tRNA was added and incubation was continued for 30 minutes. The reaction was washed 5 times with 200 μl 1x Buffer A and proteins eluted by incubation for 5 min at 95°C in 50 μl 2x SDS buffer (100 mM Tris pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM β-mercaptoethanol) and after centrifugation the supernatant was collected. In control reactions, the samples were treated as above but no RNA was immobilized on the beads. The samples were analyzed by SDS PAGE, silver stained and proteins of interest were cut out and analyzed by mass spectrometry.

In-gel digestion and mass spectrometry. The chosen silver stained gel bands were treated for in-gel digestion as described

in reference 40. Briefly, the bands were destained using Farmer's reagent and trypsin (porcine, modified, sequence grade, Promega, Madison, WI USA) was introduced into the dried gel pieces. After overnight tryptic digestion, the peptides were bound to a C18 μZipTip and after washing, eluted with acetonitrile containing matrix (alfa-cyano 4-hydroxy cinnamic acid) directly onto the target plate. The mass lists were generated by MALDI-TOF mass spectrometry on an Ultraflex III TOF/TOF from Bruker Daltonics, Bremen, Germany. The search for identity was performed by scanning the current NCBInr sequence database with the tryptic peptides using the search engine ProFound (http://prowl.rockefeller.edu/prowl-cgi/ProFound). The spectrum was internally calibrated using autolytic tryptic peptides, and the error was set at ±0.02 Da. One missed cleavage was allowed, and methionine could be oxidized. The significance of the identity was judged from the search engine's scoring system and other parameters such as from the similarity between the masses of the empiric and calculated peptides.

When required, or for confirmation, identity of a target protein was found by BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) after a fragmentation analysis was made by Post Source Decay of selected peptides.

Subcellular analysis by sucrose gradient fractionation. The gradient was prepared by mixing 5 and 50% sucrose (w/v) in 1x RB (20 mM Hepes KOH pH 7.5, 5 mM MgAc, 1 mM DTT, 150 mM KAc) with a gradient mixer. The gradient was allowed to settle at 4°C for 30 min before 1 mg cytoplasmic cell extract prepared as above in 1x RB was applied on top. Tubes were centrifuged at 20 K for 17 hours at 4°C in a Beckman SW41Ti rotor. The gradient was subsequently fractionated and collected in 0.5 ml fractions. The fractions were pooled three and three before phenol/chloroform extraction and EtOH precipitation. The samples were analyzed by northern blot for DdR-21, DdR-35/36, 5S rRNA, SRP RNA using DNA oligos GTT-47, AH-1650-A10, 5S, AA-44 respectively. A riboprobe for detecting actin mRNA was made by amplifying part of the actin gene from genomic DNA using primers 426 and 427. A T7 promoter was introduced to facilitate the in vitro transcription of the antisense strand, using P32 α-UTP (PerkinElmer) for labeling.

Protein purification. The spliced gene for CIBP, with addition of sequences for either C- or N-terminal 6-His-tags, was PCR amplified from cDNA (AX4) with primers 577 and 578, and 576 and 579, respectively, and TOPO cloned into pEXP5-CT (Invitrogen). The plasmids were transformed into BL21 AI cells (Invitrogen). Protein expression (500 ml cultures) was induced with 2 mg/ml arabinose at 18°C for 16 h. Cells were harvested at 4,500x g for 20 min and resuspended in 0.1 Tris pH 7.8. Before sonication NaCl was adjusted to 300 mM and Complete EDTA free protease inhibitor (Roche) was added. The protein was purified on a His-Trap column using an ÄKTA purifier (Pharmacia Biotech). The column was equilibrated in 50 mM Tris pH 7.8, 300 mM NaCl and after sample loading the column was washed in up to 1 M NaCl to try to get rid of *E. coli* RNA. The protein was gradient eluted with a final concentration of 300 mM Imidazol. The samples were then dialyzed to 50 mM Hepes pH 7, 100 mM NaCl and then concentrated in Vivaspin columns (GE Healthcare). It should be noted that RNAs were co-eluted with the protein indicating that the RNA binding sites were at least partly occupied. Washing the column with high salt buffers did not solve this problem. The protein precipitated at low concentration in a variety of buffers and pH making gel filtration impossible. Despite a high calculated pI, the protein also failed to bind to ion exchange columns and we decided to use the already relatively pure CIBP for gel shift assay without further purification.

Electrophoretic mobility shift assay. 50,000 cpm of 5' labeled DdR-21 RNA was denaturated and then allowed to renaturate in 1x RB (see above) for 15 min at RT. The RNA was then incubated with 500 μg/ml cell extract or 125 μg/ml purified CIBP and Ribolock RNase inhibitor (Fermentas) in 1x RB for 10 min at RT. In competition assays, yeast tRNA (Ambion) or in vitro transcribed unlabeled DdR-21 was added to the reaction and incubated for 10 min at RT. Samples were then immediately loaded on a native gel (6% rotipherose, 1x TB, 1 mM $MgCl_2$). The gels were run at 4°C at 200 V for approximately 2 h and then analyzed by a Phosphor Imager (Molecular Dynamics).

Northern blot. Northern blot analyses were performed essentially according to reference 11. Total RNA was prepared using TRIzol reagent (Invitrogen) and 10–20 μg RNA was separated on 10–12% polyacrylamide/7 M urea/1x TEB gels and electroblotted to Hybond-N^+ membranes (GE Healthcare). RNA was immobilized by UV crosslinking and analyzed by a Phosphor Imager. 5' P^{32} labeled DNA oligos were used as probes and hybridized to membranes at 42°C o/n in Church buffer and subsequently washed at 42°C.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Note:

Supplemental material can be found at
www.landesbioscience.com/journals/rnabiology/article/17214

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